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## 8-Hydroxydeoxyguanosine Causes Death of Human Leukemia Cells Deficient in 8-Oxoguanine Glycosylase 1 Activity by Inducing Apoptosis<sup>1</sup> 1 The Ministry of Science and Technology of Korea through the National Research Laboratory for Free Radicals.

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# 8-Hydroxydeoxyguanosine Causes Death of Human Leukemia Cells Deficient in 8-Oxoguanine Glycosylase 1 Activity by Inducing Apoptosis

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## Abstract

Our previous study showed that KG-1, a human acute leukemia cell line, has mutational loss of 8-oxoguanine (8-hydroxyguanine;  $\text{oh}^8\text{Gua}$ ) glycosylase 1 (OGG1) activity and that its viability is severely affected by 8-hydroxydeoxyguanosine (8-oxodeoxyguanosine;  $\text{oh}^8\text{dG}$ ). In the present study, the nature of the killing action of  $\text{oh}^8\text{dG}$  on KG-1 was investigated. Signs observed in  $\text{oh}^8\text{dG}$ -treated KG-1 cells indicated that death was due to apoptosis, as demonstrated by: increased sub-G<sub>1</sub> hypodiploid (apoptotic) cells, DNA fragmentation, and apoptotic body formation; loss of mitochondrial transmembrane potential, the release of cytochrome *c* from mitochondria into the cytosol, and the down-regulation of *bcl-2*; and the activation of caspases 8, 9, and 3, and the efficient inhibition of the apoptotic process by caspases inhibitors. This apoptosis appears not to be associated with Fas/Fas ligand because the expressions of these proteins were unchanged. Apoptotic KG-1 cells showed a high concentration of  $\text{oh}^8\text{Gua}$  in DNA. Moreover, the increased concentration of  $\text{oh}^8\text{Gua}$  in DNA, and the apoptotic process were not suppressed by the antioxidant, *N*-acetylcysteine, and thus the process is independent of reactive oxygen species. Of the 18 cancer cell lines treated with  $\text{oh}^8\text{dG}$ , 3 cell lines (H9, CEM-CM3, and Molt-4) were found to be committed to apoptosis, and all of these showed very low OGG1 activity and a marked increase in the concentration of  $\text{oh}^8\text{Gua}$  in DNA. These observations indicate that in addition to its mutagenic action,  $\text{oh}^8\text{Gua}$  in DNA disturbs cell viability by inducing apoptosis.

## Introduction

In spite of its facile formation in the presence of reactive oxygen species (ROS), knowledge of the action of 8-hydroxyguanine (8-oxoguanine;  $\text{oh}^8\text{Gua}$ ) in DNA is limited to its mutagenicity. Moreover, even this action is the result of speculation based on *in vitro* observations, such as, the mismatch of  $\text{oh}^8\text{Gua}$  with A instead of C and the resulting GC→TA transversion. These relationships are demonstrated by a DNA polymerase reaction using an  $\text{oh}^8\text{Gua}$  containing an oligonucleotide template (1, 2), or by indirect *in vivo* data, such as the high or low mutation rates observed in cells with a deficiency or a supplementation of  $\text{oh}^8\text{Gua}$ 's repair enzyme, which is known as 8-oxoguanine glycosylase 1 (OGG1) (3, 4). Nevertheless, many chemicals or forms of radiation that produce ROS cause various cytotoxic changes in cells and increase the  $\text{oh}^8\text{Gua}$  level in DNA. Thus, some of these cytotoxic changes are expected to be mediated by the  $\text{oh}^8\text{Gua}$  in DNA formed by generated ROS (5, 6). However, conclusive evidence of this mediation is unavailable because such ROS-producing agents cause modifications in DNA other than the production of  $\text{oh}^8\text{Gua}$ , and, therefore, the exclusive formation of  $\text{oh}^8\text{Gua}$  in cellular DNA has never been observed.

KG-1 is an acute leukemia cell line, which has no OGG1 activity because of a mutation in the OGG1 gene. In a previous study, we found that this cell line when treated with 8-hydroxydeoxyguanosine (a nucleoside of  $\text{oh}^8\text{Gua}$ ;  $\text{oh}^8\text{dG}$ ), shows a high level of  $\text{oh}^8\text{Gua}$  in its DNA and dies (7). This result suggests that the death of  $\text{oh}^8\text{dG}$ -treated KG-1 is due to an increased level of  $\text{oh}^8\text{Gua}$  in DNA and implies that  $\text{oh}^8\text{Gua}$  in DNA can be lethal. In the present study, we attempted to elucidate the mode of action of  $\text{oh}^8\text{Gua}$  in DNA by studying the nature of the  $\text{oh}^8\text{dG}$  that induces the death of KG-1, and to determine the relation between the cell-killing action of  $\text{oh}^8\text{dG}$  and OGG1 deficiency. Our results show that  $\text{oh}^8\text{dG}$ -induced cell death is mediated by an apoptotic process and we suggest that this is another function of  $\text{oh}^8\text{Gua}$  in DNA.

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## Results

### Death of KG-1 Cells and Increased $oh^8Gua$ Level in DNA After $oh^8dG$ Treatment

KG-1 cells deficient in OGG1 activity were treated with  $oh^8dG$ , and their viability and  $oh^8Gua$  levels in DNA were determined. As shown in Fig. 1, the viability of KG-1 cells, as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test, was severely affected by

$oh^8dG$  in a time-dependent (Fig. 1A) and dose-dependent (Fig. 1B) manner. After 96 h incubation, 50% of KG-1 cells were killed by 50  $\mu g/ml$  of  $oh^8dG$  (Fig. 1B). At the same time, the concentration of  $oh^8Gua$  in DNA increased in a time- and dose-dependent fashion (Fig. 1C). In contrast, U937 cell line with normal OGG1 activity showed almost no changes in viability or in  $oh^8Gua$  level in DNA.

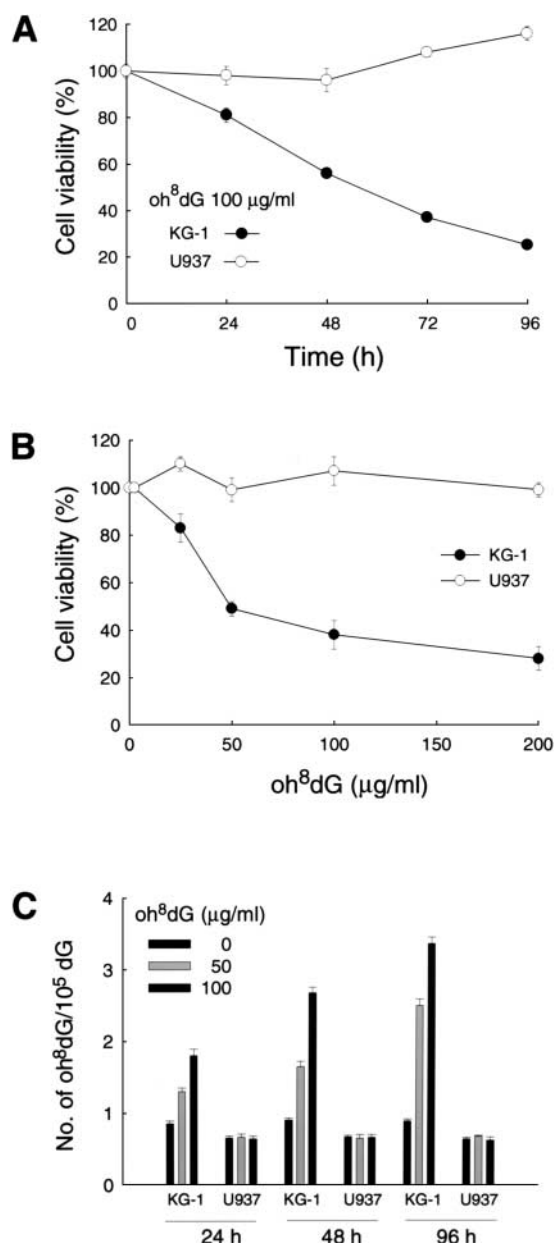
### Induction of Apoptosis in $oh^8dG$ -Treated KG-1 Cells

To determine the cause of KG-1 death by  $oh^8dG$  (Fig. 1, A and B), apoptosis was examined in different ways. First, the appearance of sub- $G_1$  hypodiploid cells, which are known to be apoptotic cells (8), was examined by flow cytometry. As shown in Fig. 2A, the proportion of these apoptotic cells in  $oh^8dG$ -treated KG-1 cells dramatically increased in a time- and dose-dependent manner. Apoptotic body formation (Fig. 2B) was also observed microscopically and through DNA fragmentation by electrophoresis (Fig. 2C), both of which are indicators of apoptosis (9). Here again, U937 showed almost no changes in terms of these three criteria, *i.e.*, the number of sub- $G_1$  hypodiploid cells, apoptotic body formation, and DNA fragmentation.

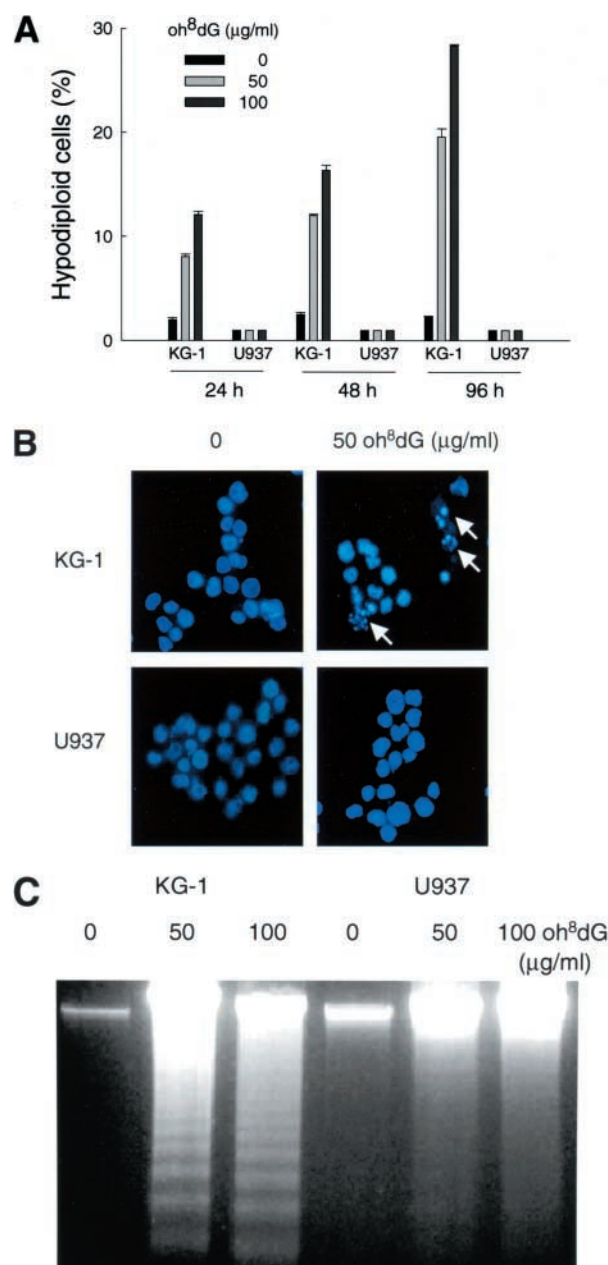
### Changes in Proteins Involved in the Apoptotic Process

To understand the mechanistic background of  $oh^8dG$ -induced apoptosis, we first examined changes in the amounts of Bax and Bcl-2 by Western blot. As shown in Fig. 3, the amount of Bax, a pro-apoptotic protein, showed no change. However, Bcl-2, an anti-apoptotic protein, decreased gradually with time. One of the actions of Bcl-2 during the apoptotic process is to prevent the opening of mitochondrial membrane pores (10). It is suggested that pore opening induces the disruption of  $\Delta\phi_m$ , which in turn leads to the release of cytochrome *c* from mitochondria (11). Therefore, the low level of Bcl-2 (Fig. 3) may augment these mitochondrial events in  $oh^8dG$ -treated KG-1 cells. Fig. 4A shows the increased proportion of cells with disrupted  $\Delta\phi_m$  versus time after KG-1 cells were treated with  $oh^8dG$ . Interestingly, the kinetics of cell increase versus a loss of  $\Delta\phi_m$  (Fig. 4A) coincided precisely with the increase of apoptotic sub- $G_1$  hypodiploid cells (Fig. 4C). Fig. 4B shows a decreased level of cytochrome *c* in mitochondria and a simultaneous increase of its level in the cytosol, indicating a translocation of cytochrome *c* from mitochondria to the cytosol.

The observed loss of  $\Delta\phi_m$  and of cytochrome *c* release in mitochondria led us to examine the status of caspases, because they are known to link proximal and distal mitochondrial apoptotic events. First, caspase 8 was examined, as this acts proximally to apoptotic mitochondria (12). Western blot showed an increase in the active form of caspase 8 ( $M_r$  20,000) (Fig. 5A), which was confirmed by enzyme assay (Fig. 5B). Consistent with these results, Bid, its substrate protein, was found to have been cleaved ( $M_r$  18,000) (Fig. 5A), which is known to disrupt  $\Delta\phi_m$  to release cytochrome *c* by opening the mitochondrial membrane pores (13). Thus, the cleavage of Bid (Fig. 5A) is a phenomenon that is synergistic with Bcl-2 decline (Fig. 3). We next examined caspase 9 by Western blot because it is known that this enzyme is activated



**FIGURE 1.** Effect of  $oh^8dG$  on the viability of KG-1 cells and  $oh^8Gua$  levels in DNA. KG-1 cells were cultured for various times with 100  $\mu g/ml$  of  $oh^8dG$  (A) or for 96 h in the presence of various concentrations of  $oh^8dG$  (B). After harvesting, cells were examined for viability using the MTT test. At the same time, DNA was isolated from the cells and the amount of  $oh^8Gua$  (C) was determined ( $n = 3$ ). Details are described in "Materials and Methods."



**FIGURE 2.** Apoptosis of KG-1 cells by oh<sup>8</sup>dG. Cells were cultured with oh<sup>8</sup>dG and examined for evidence of apoptosis using different methods. **A.** Flow cytometry for the measurement of sub-G<sub>1</sub> hypodiploid cells ( $n = 3$ ). **B.** Microscopy which demonstrated apoptotic bodies after incubating with oh<sup>8</sup>dG at 50 µg/ml for 96 h. **C.** Electrophoresis for the detection of DNA fragmentation after incubating with oh<sup>8</sup>dG at 50 µg/ml for 96 h. Details are described in "Materials and Methods."

by cytochrome *c* in the cytoplasm (14). As expected, the active form ( $M_r$  37,000) of caspase 9 was increased by Western blot (Fig. 5A) in parallel with its activity (Fig. 5C). Finally, we examined caspase 3, a target of caspase 9, and, as shown in Fig. 5A, the expressions of the two active forms ( $M_r$  12,000 and 17,000) of caspase 3 increased, which was further demonstrated by the cleavage of poly ADP-ribosyl polymerase (PARP) from  $M_r$  115,000 to 85,000. These results indicate

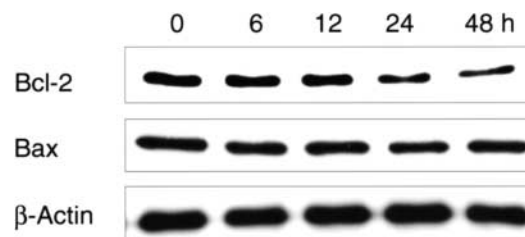
that the apoptosis of KG-1 induced by oh<sup>8</sup>dG is caspase dependent. This dependency was further examined by treating KG-1 cells with oh<sup>8</sup>dG in the presence of benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), a general caspase inhibitor (15) and benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD-fmk), a selective inhibitor of caspase 3 (16). As shown in Fig. 6, these inhibitors suppressed the formation of sub-G<sub>1</sub> hypodiploid cells completely (Fig. 6A) and at the same time blocked the activation of caspase 3, as indicated by the formation of its active forms ( $M_r$  12,000 and 17,000) and the cleavage of its substrate PARP (Fig. 6B).

#### No Involvement of the Fas System and ROS

Of the caspases examined, caspase 8 was identified as the most proximally acting enzyme. Several factors are associated with the control of caspase 8 activity, and the Fas system has been proposed as a major activator (17). Thus, the involvement of the Fas system was tested by examining the expressions of Fas and Fas ligand (Fas L) in oh<sup>8</sup>dG-treated KG-1 cells. However, Fig. 7A shows no change in the number of Fas-positive cells by flow cytometry, and Fig. 7B shows no change in Fas-L by Western blot. These results indicate a low likelihood of Fas system involvement. Another possibility examined was that ROS is involved in oh<sup>8</sup>dG-induced apoptosis. Recently, oh<sup>8</sup>dG was found to be very reactive toward ROS (18), which suggested that oh<sup>8</sup>dG might produce ROS during its degradation in cells. It is also known that ROS can directly induce apoptosis (19, 20). In fact, some ROS have been shown to be produced (Fig. 8A) by the action of oh<sup>8</sup>dG on KG-1, but the level of ROS was too small to induce cytotoxicity, because NAC was unable to prevent the death of KG-1, as assessed by the MTT test (Fig. 8B), or apoptosis as determined by the appearance of sub-G<sub>1</sub> hypodiploid cells (Fig. 8C).

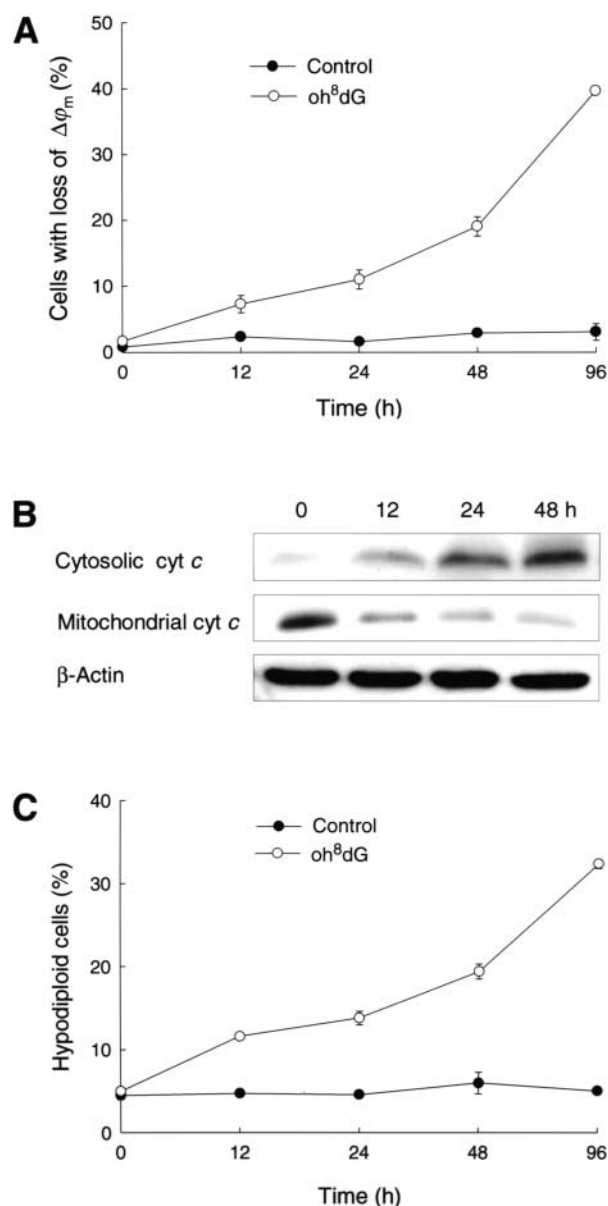
#### The Relevance of oh<sup>8</sup>dG Toxicity to Low OGG1 Activity

In contrast to the KG-1 cells, U937, which is normal in terms of OGG1 activity, was totally resistant to oh<sup>8</sup>dG, suggesting a relation between the observed susceptibility to oh<sup>8</sup>dG and low OGG1 activity. To test this possibility, 18 cell lines were screened for viability using the MTT test after they had been treated with various concentrations of oh<sup>8</sup>dG for 96 h.



**FIGURE 3.** Effect of oh<sup>8</sup>dG on the expressions of Bcl-2 and Bax in KG-1 cells. Cells were cultured with 50 µg/ml of oh<sup>8</sup>dG for the indicated times and subjected to electrophoresis for Western blot using mouse monoclonal anti-Bcl-2, -Bax, or -β-actin as a primary antibody and goat anti-mouse IgG conjugated with horseradish peroxidase as a secondary detection antibody. Details are described in "Materials and Methods."





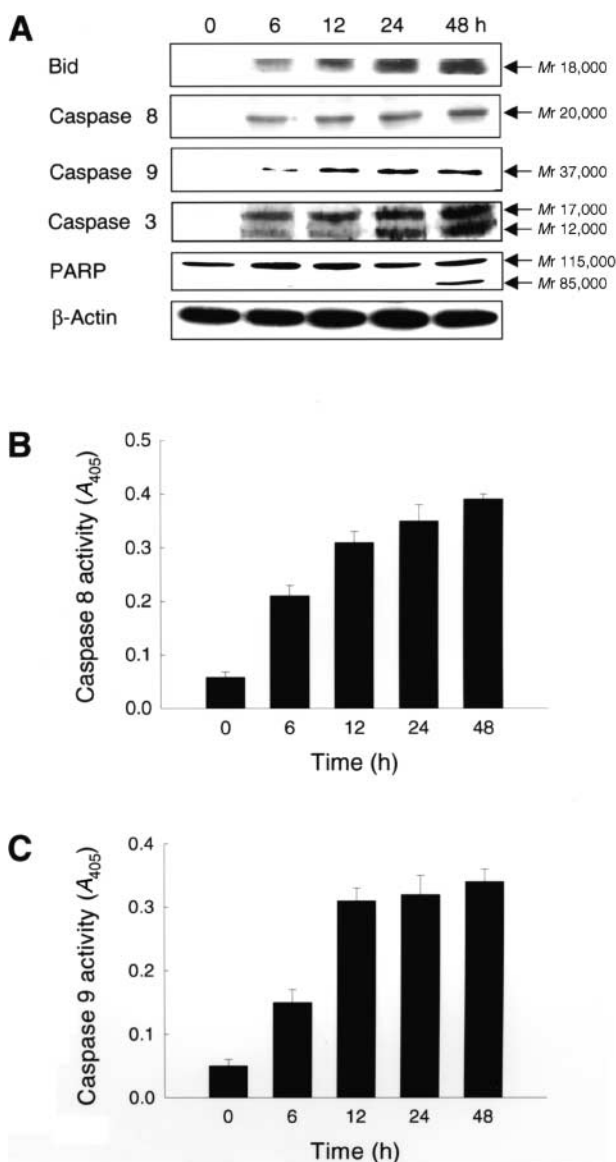
**FIGURE 4.** Loss of mitochondrial membrane potential ( $\Delta\psi_m$ ) and the release of cytochrome c from mitochondria into the cytosol in KG-1 cells treated with  $oh^8dG$ . KG-1 cells ( $2 \times 10^5$  cells/ml) were treated with 50  $\mu$ g/ml of  $oh^8dG$  for the times indicated. **A**, The cells harvested were incubated with 30 mM of DiOC<sub>6</sub>(3) for 15 min at 37°C and then subjected to flow cytometry, which used the fluorescence intensity of this fluorochrome ( $n = 4$ ). **B**, KG-1 cells ( $2 \times 10^5$  cells/ml) were treated with 50  $\mu$ g/ml of  $oh^8dG$  for the times indicated. The harvested cells were fractionated into mitochondria and cytosol, as described in "Materials and Methods." Aliquots of each fraction were subjected to Western blot for cytochrome c (cyt c). **C**, Cells treated as in **A** were analyzed for sub-G<sub>1</sub> hypodiploid cells by flow cytometry ( $n = 3$ ).

In addition to KG-1, another three susceptible cell lines were found, *i.e.*, CEM-CM3, Molt-4, and H9 (Fig. 9A). The sensitive cell lines proved as sensitive as KG-1, while others showed no response to 200  $\mu$ g/ml of  $oh^8dG$ . The sensitive cell lines also showed very low OGG1 activities (Fig. 9B); however, they showed no mutation of the OGG1 gene, reduced OGG1 protein expression (data not shown), or an increased

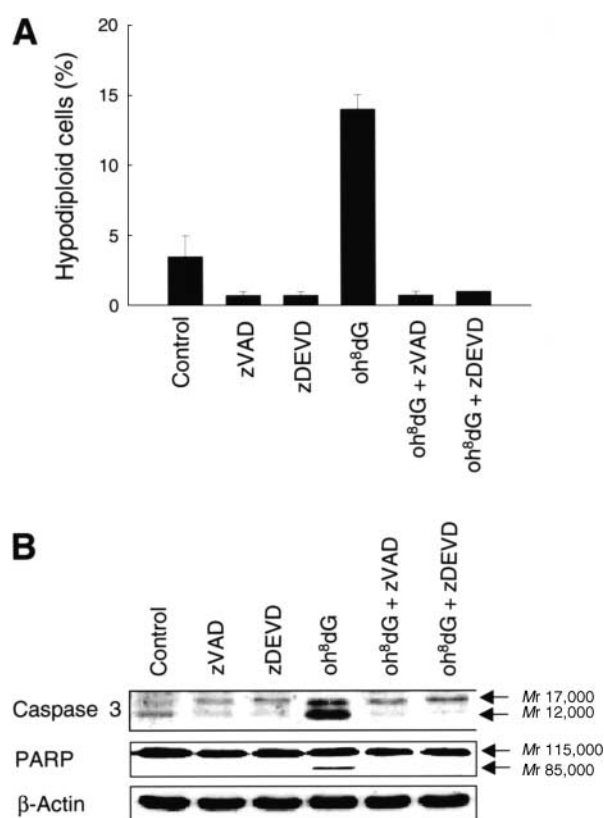
amount of  $oh^8Gua$  in their DNA (Fig. 9C). Moreover, the enzyme activity and the  $oh^8Gua$  levels in the DNA of resistant cell lines were almost the same as that of U937, as shown in Fig. 9, B and C (data not shown).

## Discussion

The present study demonstrates that  $oh^8dG$  induces apoptotic cell death in cells with low OGG1 activity, and that the concentration of  $oh^8Gua$  is increased in the DNA of such cells. In view of the fact that DNA-damaging agents have been observed to induce apoptosis (6), the elevation of  $oh^8Gua$  in DNA might be expected to trigger apoptosis. These observations indicate that in addition to its mutagenic action,  $oh^8Gua$  in



**FIGURE 5.** Activation of caspases in KG-1 cells treated with  $oh^8dG$ . KG-1 was treated with 50  $\mu$ g/ml of  $oh^8dG$  for the indicated times and used for Western blot of caspases and PARP (**A**) and caspase assays ( $n = 3$ ); caspase 8 (**B**); and caspase 9 (**C**). Details are described in "Materials and Methods."

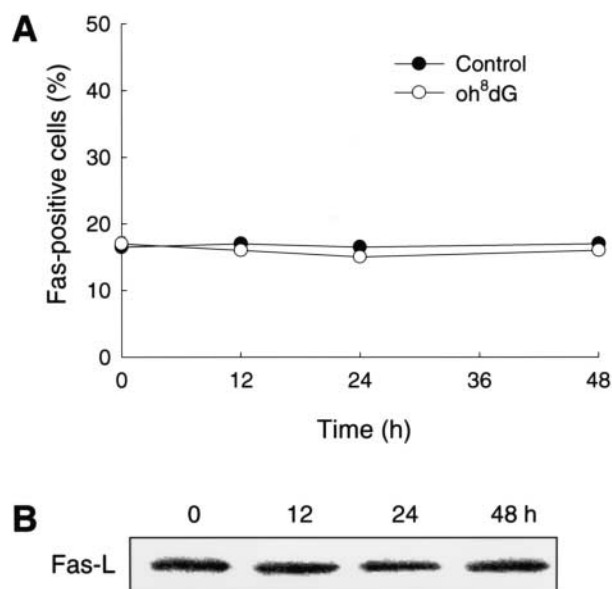


**FIGURE 6.** Effect of caspase inhibitors on the apoptosis of KG-1 cells treated with oh<sup>8</sup>dG. KG-1 was treated with oh<sup>8</sup>dG (0 or 50 μg/ml) for 48 h in the absence or presence of the caspase inhibitors, zVAD-fmk (50 μM), and zDEVD-fmk (100 μM). After harvesting, KG-1 was subjected to flow cytometric analysis for sub-G<sub>1</sub> hypodiploid cells (**A**) (*n* = 3) and to Western blot for caspase 3 and PARP (**B**). Details are described in "Materials and Methods."

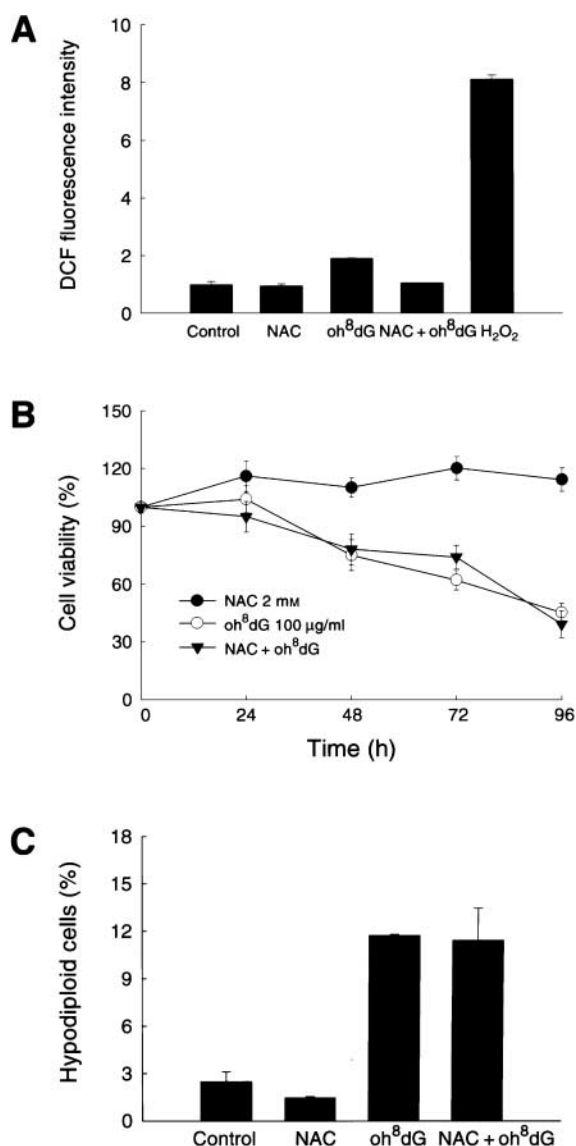
DNA disturbs cell viability and that this may be lethal. However, the disturbance of cell viability by increased oh<sup>8</sup>Gua levels, as observed in the present study, is in conflict with a report (21) that showed that the organs of OGG1-deficient mice showed no exacerbation of toxic symptoms *versus* wild-type mice at increased oh<sup>8</sup>Gua levels. Thus, the possibility that our results might reflect an idiosyncrasy of the *in vitro* system used cannot be ruled out. In addition, the oh<sup>8</sup>Gua level in mitochondrial DNA should be considered because OGG1 is known to play an important role in the removal of oh<sup>8</sup>Gua from mitochondrial DNA, thus leading to a disruption of mitochondrial activity, which again is an important aspect of the apoptotic process (22).

The apoptosis induced by DNA-damaging agents occurs via two pathways: p53-dependent activation via Fas (23–25) and/or mitochondria-mediated caspase activation (26, 27). The apoptosis induced by oh<sup>8</sup>dG appears to take place via the latter pathway, because the p53 gene is inactive in KG-1 due to mutation (28, 29) and because its expression is not changed in oh<sup>8</sup>dG-treated KG-1 (data not shown). In addition, oh<sup>8</sup>dG-treated KG-1 showed no change in the expression of Fas or Fas-L (Fig. 7). On the other hand, various biochemical findings observed in KG-1 treated with oh<sup>8</sup>dG coincide with those that have been reported in mitochondria-involved apoptosis.

Following are the major biochemical findings observed in the mitochondria of apoptotic cells. The first observed event is a permeability change of the mitochondrial membrane, which results in the release of cytochrome *c* to the cytosol (30–33). Cytochrome *c* in the cytosol complexes with Apaf-1 and procaspase 9 leading to the conversion of procaspase 9 into caspase 9, which in turn activates procaspase 3 to become caspase 3 (12). Caspase 3 then activates endonuclease causing DNA fragmentation (34). Several factors can alter the status of the mitochondrial membrane, and caspase 8 is probably the most active of these, which acts by cleaving Bid, a proapoptotic member of the Bcl-2 protein family (13, 35). The cleaved Bid opens the mitochondrial membrane pores, leading to a permeability transition (PT), which decreases  $\Delta\phi_m$ , and eventually to the release of cytochrome *c* into the cytosol (12). Bcl-2 prevents this mitochondrial membrane PT (11), whereas Bax induces PT (36). In the present study, apoptotic KG-1 provided data supporting the above cascade. Specially, this included the following observations: (a) a decline of Bcl-2 expression (Fig. 3); (b) the activation of caspase 8 (Fig. 5); (c) the cleavage of Bid (Fig. 5); (d) decreased  $\Delta\phi_m$  and cytochrome *c* in mitochondria and a commensurate increase in the cytosol (Fig. 4); and (e) the activation of caspases 9 and 3 (Fig. 5). Notably, other caspase inhibitors ceased KG-1 apoptosis by oh<sup>8</sup>dG (Fig. 6) almost completely. As opposed to what was expected, we found that the oh<sup>8</sup>dG level in KG-1 was slightly higher than that in U937, which has OGG1 activity (see Fig. 1C), which indicates that enzymes other than OGG1 may influence the oh<sup>8</sup>dG level. In contrast to OGG1, KG-1 showed normal activities of MYH and MTH1, which are two



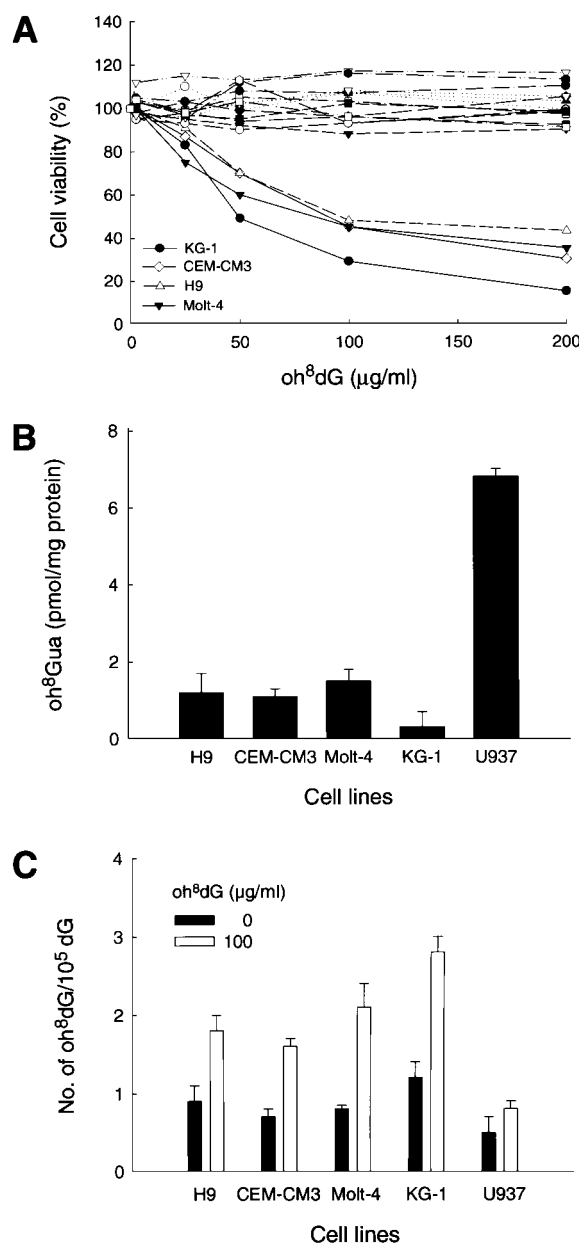
**FIGURE 7.** Effect of oh<sup>8</sup>dG on the expressions of Fas and Fas-L in KG-1 cells treated with oh<sup>8</sup>dG. KG-1 was treated in duplicate with 50 μg/ml of oh<sup>8</sup>dG for the indicated times. One portion of the treated KG-1 was incubated with FITC-conjugated mouse monoclonal anti-human Fas and subjected to the flow cytometric analysis for Fas by FITC fluorescence (**A**) (*n* = 3). The other portion was subjected to Western blot for Fas-L (**B**).



**FIGURE 8.** The involvement of ROS in the cytotoxicity and apoptosis of KG-1 treated with oh<sup>8</sup>dG. **A.** KG-1 was incubated with either oh<sup>8</sup>dG (100 µg/ml), H<sub>2</sub>O<sub>2</sub> (200 µM), or NAC (2 mM) for 96 h at 37 °C, and incubated further in the presence of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 100 µM) for 1 h at 37 °C in the dark. The fluorescence of 2',7'-dichlorodihydrofluorescein (DCF), an oxidation product of DCFH-DA, was then measured. **B.** KG-1 was incubated with oh<sup>8</sup>dG (100 µg/ml), NAC (2 mM), or both for the times indicated at 37 °C and then subjected to an MTT test. **C.** KG-1 was treated with oh<sup>8</sup>dG (100 µg/ml), NAC (2 mM), or both for 96 h and subjected to flow cytometric analysis for sub-G<sub>1</sub> hypodiploid cells. Experiments were performed in triplicate. Details are described in "Materials and Methods."

enzymes involved in repair of oh<sup>8</sup>dG (data not shown). The former (37) removes adenine to mismatch to oh<sup>8</sup>dG, and the latter (38) hydrolyzes oh<sup>8</sup>dGTP to oh<sup>8</sup>dGMP and thus, prevents its incorporation into DNA. In addition, comparisons should be made of the cell viabilities and oh<sup>8</sup>dG accumulations of cells with the same genetic background with the exception of OGG1, for example, KG-1 and KG-1 expressing wild-type OGG1. In fact, a significant amount of time was spent unsuccessfully trying to create KG-1 clones

of the latter type. KG-1 has impaired p53 activity owing to the mutation of this gene (28, 29). Another laboratory has made similar unsuccessful efforts to clone KG-1 expressing wild-type p53 (39). KG-1 has a property to resist the entry of exogenous DNA because in both laboratories, failure occurred at transfection. Attempts are now being made by using the antisense technique and a clone with deficient OGG1 from NIH3T3.



**FIGURE 9.** The relevance of oh<sup>8</sup>dG toxicity to low OGG1 activity. **A.** Eighteen cell lines including KG-1 (see "Materials and Methods") were incubated with various concentrations of oh<sup>8</sup>dG for 96 h, and their viability was then determined using the MTT test. Of the 18 cell lines, 4 cell lines, including KG-1, found to be susceptible to oh<sup>8</sup>dG, and U937 cells, resistant to oh<sup>8</sup>dG, were assayed for OGG1 (**B**) and oh<sup>8</sup>Gua levels in their DNA (**C**). Experiments were performed in triplicate. Details are described in "Materials and Methods."

Recently, we found that KG-1 and other cell lines with low OGG1 activity show much higher sensitivity to radiation than cells with normal OGG1 activity (6). On being exposed to  $\gamma$  irradiation, these cells show a higher concentration of  $\text{oh}^8\text{Gua}$ -treated cells in their DNA and undergo apoptosis. This means that the impaired viability of  $\text{oh}^8\text{dG}$  causes an elevation of the  $\text{oh}^8\text{Gua}$  level in the DNA (Fig. 1C), and also strongly suggests that  $\text{oh}^8\text{Gua}$  in the DNA is cytotoxic enough to kill cells. Thus, cytotoxicity induced by either ROS-producing agents or cell damage observed in the pathological conditions accompanying high oxidative stress may contribute to this action of  $\text{oh}^8\text{Gua}$  in DNA.

It is not understood, however, how the concentration of  $\text{oh}^8\text{Gua}$  is elevated in the DNA of cells with low OGG1 activity, as shown in Figs. 1C and 9C. This is prompted by reports that  $\text{oh}^8\text{dGDP}$ , a substrate of DNA polymerase, is not formed from  $\text{oh}^8\text{dGMP}$ , because guanylate kinase, which makes dGDP from dGMP, does not use  $\text{oh}^8\text{dGMP}$  as a substrate (40, 41). And by our observations that  $^{14}\text{C}$ -labeled  $\text{oh}^8\text{dG}$  was found not to be incorporated into DNA, even though it was incorporated into cytosol (data not shown). We observed that polymerase  $\beta$ , which can incorporate dNTP with a modified base into DNA (42) was expressed at a higher level in KG-1 than in U937, after these cells had been treated with  $\text{oh}^8\text{dG}$ . This increased expression of polymerase  $\beta$  may be a reason for the elevation of the  $\text{oh}^8\text{Gua}$  level in the DNA of KG-1, because both polymerase  $\beta$  expression and the elevation of  $\text{oh}^8\text{Gua}$  levels were suppressed in the presence of deoxythymidine, an inhibitor of polymerase  $\beta$  (43) (data not shown). The possible involvement of ROS as an effector of  $\text{oh}^8\text{Gua}$  elevation in DNA was excluded by the finding that an antioxidant, NAC, has no effect on  $\text{oh}^8\text{dG}$ -induced apoptosis (Fig. 8).

This study demonstrates that  $\text{oh}^8\text{dG}$  is able to elevate the level of  $\text{oh}^8\text{Gua}$  in DNA, but only in those cells with low OGG1 activity. It further shows that cells with high levels of  $\text{oh}^8\text{Gua}$  in DNA die because of apoptosis triggered by these elevated  $\text{oh}^8\text{Gua}$  levels. From these results, it appears that  $\text{oh}^8\text{Gua}$  in DNA is toxic enough to kill cells and that  $\text{oh}^8\text{dG}$  exerts a selective lethality in OGG1-deficient cells. This toxicity of  $\text{oh}^8\text{Gua}$  in DNA may provide a mechanistic basis for understanding the toxicities of many ROS-producing agents and of the cell damage observed in many ROS-involved clinical conditions. Moreover, this action may provide a background explanation for the beneficial effects of various antioxidants. Finally, the lethal action of  $\text{oh}^8\text{dG}$  may have applications in the selective chemotherapy of cancers with deficient OGG1 activity because of its lack of toxicity to normal cells.

## Materials and Methods

### Reagents

8-Hydroxydeoxyguanosine ( $\text{oh}^8\text{dG}$ ), MTT, propidium iodide, RNase, and Hoechst 33258 dye were purchased from Sigma Chemical Co. (St. Louis, MO); the caspase inhibitors, zVAD-fmk, zDEVD-fmk and the antioxidant, *N*-acetylcysteine (NAC), were from Calbiochem (La Jolla, CA); and DCFH-DA was from Molecular Probes (Eugene, OR). All other reagents, unless indicated, were from Sigma.

### Cell Culture

KG-1 (human acute myelocytic leukemia cell line), U937 (human monocytic leukemia cell line), SNU-C4 (human colorectal adenocarcinoma cell line), Hep G2 (human hepatoblastoma cell line), SNU-1, and Kato III (human gastric cell lines), IM-9 (human melanoma cell line), Molt-4, H9, Jurkat, CCRF-CEM (human T lymphoblastic leukemia cell lines), K562 (human erythrocytic leukemia cell line), and Jiyoye (human B lymphocytic leukemia cell line) were cultured at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  in RPMI 1640 containing 2 mM glutamine, 10% heat-inactivated FCS, penicillin (100 units/ml), and streptomycin (100  $\mu\text{g}/\text{ml}$ ) in the absence or presence of various concentrations of  $\text{oh}^8\text{dG}$  and/or other chemicals. SK-N-SH, SH-SY5Y, and SK-N-BE(2)C (human neuroblastoma cell lines) and CEM-CM3 and CCRF-HSB2 (human T lymphoblastic leukemia cell lines) were cultured under exactly the same condition as mentioned immediately above but DMEM was used instead of RPMI 1640.

### Cell Viability Assay

The effect of  $\text{oh}^8\text{dG}$  on the viability of various cancer cell lines was determined by MTT assay (44). Cells at the exponential phase were collected and transferred into each well (about  $10^4$ – $10^5$  cells in 180  $\mu\text{l}$ /well). The cells were incubated for various times (up to 96 h) in the presence of various amounts of  $\text{oh}^8\text{dG}$  (0–200  $\mu\text{g}/\text{ml}$ ) in a total reaction volume of 200  $\mu\text{l}$ , and 50  $\mu\text{l}$  of 2 mg/ml MTT solution was then added to each well (0.1 mg/well). After incubating for 4 h, the plates were centrifuged at  $800 \times g$  for 5 min and supernatants were aspirated. The formazan crystals in each well were dissolved in 150  $\mu\text{l}$  of DMSO and the  $A_{540}$  was read on a scanning multi-well spectrophotometer (Molecular Device Co., Sunnyvale, CA). All experiments were performed in triplicate.

### Flow Cytometry

Cells were treated with  $\text{oh}^8\text{dG}$ , as above, in the absence or presence of various compounds, and flow cytometry was performed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). To examine the induction of apoptosis by  $\text{oh}^8\text{dG}$ , we investigated the formation of sub- $G_1$  hypodiploid cells (apoptotic cells). Cells ( $2 \times 10^5$  cells/ml) were cultured with  $\text{oh}^8\text{dG}$  (0–100  $\mu\text{g}/\text{ml}$ ) in the absence or in the presence of zVAD-fmk (50  $\mu\text{M}$ ), zDEVD-fmk (100  $\mu\text{M}$ ), or NAC (2 mM), harvested at the times indicated and fixed in 1 ml of 70% ethanol. The cells ( $2 \times 10^5/\text{ml}$ ) in ethanol solution were washed twice with PBS and then incubated in the dark in 1 ml of PBS containing 100  $\mu\text{g}$  of propidium iodide for 30 min at  $37^\circ\text{C}$ . The proportion of sub- $G_1$  hypodiploid cells was determined by using histograms generated by the Cell Quest and Mod-Fit computer programs (8). To measure Fas expression, cells ( $2 \times 10^5/\text{ml}$ ) were treated with 50  $\mu\text{g}/\text{ml}$  of  $\text{oh}^8\text{dG}$ , harvested at various times and washed twice with PBS. The washed cells were then incubated for 2 h on ice with 20  $\mu\text{l}$  of FITC-conjugated mouse monoclonal antibody to human Fas (DX-2; PharMingen, San Diego, CA), washed with PBS, and subjected to flow cytometry for Fas expression by FITC fluorescence. To determine the mitochondrial membrane



potential ( $\Delta\psi_m$ ), cells ( $2 \times 10^5$  cells/ml) were treated with 50  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  for various times, as shown in Fig. 4, and incubated with 30 nM of  $\text{DiOC}_6(3)$  (Molecular Probes), a cationic lipophilic fluorochrome, for 15 min at  $37^\circ\text{C}$ , and subjected to flow cytometry to determine the cell distribution by  $\text{DiOC}_6(3)$  fluorescence (10).

#### Fluorescence Microscopy and Electrophoresis

The induction of apoptosis by  $\text{oh}^8\text{dG}$  was also examined morphologically and electrophoretically. For the former, the same cells ( $2 \times 10^5/\text{ml}$ ) treated with 50  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  for 48 h were washed with PBS and fixed in 4% paraformaldehyde for 5 min. The cells were then stained with Hoechst 33258 dye (8  $\mu\text{g/ml}$ ) for 5 min, washed twice with PBS, and mounted. Nuclei were observed using an Axiovert 100 inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany) (9). For electrophoretic examination, the same cells ( $2 \times 10^5/\text{ml}$ ) as treated above were washed with PBS, and DNA was extracted using a genomic DNA extraction kit (Wako junyaku, Tokyo, Japan). Aliquots of the DNA (4  $\mu\text{g}$ ) dissolved in 5  $\mu\text{l}$  of a loading buffer were electrophoresed in a 1.8% agarose gel containing 0.1  $\mu\text{g/ml}$  of ethidium bromide.

#### Western Blot Analysis

KG-1 ( $2 \times 10^5$  cells/ml) cells cultured with 50  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  in the absence or in the presence of caspase inhibitors were harvested at the indicated times. Cells were lysed by boiling for 5 min in 500  $\mu\text{l}$  of a solution of 120 mM NaCl, 0.1% NP40, and 40 mM Tris-HCl (pH 8.0). Aliquots of the lysates (40  $\mu\text{g}$  of protein) were electrophoresed on a 10% SDS-polyacrylamide gel, and the gel was transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was then incubated with primary mouse monoclonal anti-human caspase 3, -8, -9, and -PARP, -Bcl-2, -Bax, -Bid, -Fas ligand (Fas-L), and - $\beta$ -actin antibodies (PharMingen) and then with a secondary goat anti-mouse IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL). The membrane was then exposed to X-ray film, and the protein bands were detected by using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

For the detection of mitochondrial and cytosolic cytochrome *c*, KG-1 cells ( $2 \times 10^5$  cells/ml) were cultured with 50  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  and harvested at the times indicated in Fig. 4. The cells were suspended in 50  $\mu\text{l}$  of ice-cold homogenation buffer [250 mM sucrose, 10 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES (pH 7.5)] and homogenized in a Teflon homogenizer. The homogenate was centrifuged at  $750 \times g$  for 10 min at  $4^\circ\text{C}$ , and the supernatant obtained was centrifuged at  $10,000 \times g$  for 15 min at  $4^\circ\text{C}$ . The resulting pellet was resuspended in homogenation buffer and used as a mitochondrial fraction. The supernatant obtained by centrifuging at  $10,000 \times g$  for 15 min above was further centrifuged at  $10,000 \times g$  for 60 min at  $4^\circ\text{C}$  and the supernatant collected was used as a cytosolic fraction. The mitochondrial and cytosolic fractions were analyzed by Western blot for cytochrome *c* as described above using a mouse monoclonal anti-human cytochrome *c* antibody (PharMingen) (45).

#### Caspases 8 and 9 Assays

KG-1 cells ( $2 \times 10^5$  cells/ml) were cultured with 50  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  for various times and harvested. The cells were used to assay caspases 8 and 9 by using a colorimetric kit (for caspase 8, Clontech Laboratories, Inc., Palo Alto, CA; and for caspase 9, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The chromophore used in both cases was *p*-nitroaniline (pNA), which was released from IETD-pNA by caspase 8 and from LEHD-pNA by caspase 9.

#### ROS Measurements

To determine the involvement of ROS in the induction of cytotoxicity by  $\text{oh}^8\text{dG}$ , cells were treated with 100  $\mu\text{g/ml}$  of  $\text{oh}^8\text{dG}$  for 96 h and then further incubated in the presence of 100  $\mu\text{M}$  of DCFH-DA in the dark for 1 h at  $37^\circ\text{C}$ . To observe the effect of the antioxidant, NAC, the reaction was performed under the same conditions, but the cells were preincubated with 2 mM of NAC for 30 min before the  $\text{oh}^8\text{dG}$  treatment. As a positive control, cells were treated with 200  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  instead of  $\text{oh}^8\text{dG}$ . The fluorescence of 2',7'-dichlorofluorescein, an oxidation product of DCFH-DA, was measured using a FACSCalibur flow cytometer (Becton Dickinson) (46).

#### $\text{oh}^8\text{Gua}$ Assay

Cells ( $2 \times 10^5$  cells/ml) cultured with  $\text{oh}^8\text{dG}$  (0–100  $\mu\text{g/ml}$ ) as described above were harvested at the indicated times. DNA was extracted from these cells by using a genomic DNA extraction kit (Wako junyaku) and the amount of  $\text{oh}^8\text{Gua}$  present in the DNA was measured as  $\text{oh}^8\text{dG}$  by using a high-performance liquid chromatography (HPLC)/electrochemical detector as described previously (47). The  $\text{oh}^8\text{dG}$  level was expressed as the number of  $\text{oh}^8\text{dG}/10^5$  deoxyguanosine (No. of  $\text{oh}^8\text{dG}/10^5$  dG).

#### $\text{oh}^8\text{Gua}$ Glycosylase 1 Assay

To identify OGG1-deficient human leukemia cell lines, various cell lines were cultured as described in the previous section and screened for OGG1 activity. OGG1 was assayed using its glycosylase activity by quantifying the  $\text{oh}^8\text{Gua}$  released from substrate DNA into the reaction medium as described previously (44). Cells at the exponential phase were centrifuged at  $800 \times g$  for 5 min. Cell pellets ( $10^6$  cells/assay) were suspended in 2 volumes of homogenation buffer [50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 5% glycerol, and 0.05% 2-mercaptoethanol (pH 7.5)], and homogenized. Homogenates were mixed with streptomycin (final concentration, 1.5%) to remove nucleic acids, and supernatants, obtained by centrifugation, were dialyzed extensively against a homogenation buffer and used as cell extracts for the  $\text{oh}^8\text{Gua}$  glycosylase assay. To prepare the substrate for  $\text{oh}^8\text{Gua}$  glycosylase, a 21-mer oligonucleotide containing  $\text{oh}^8\text{Gua}$  (5'-CAGCC-AATCAGTG\*CACCATTC-3': G\*;  $\text{oh}^8\text{Gua}$ ) was chemically synthesized (The Midland Certified Reagent Co., Medland, TX), and annealed with its complementary oligonucleotide. The duplex DNA so obtained was used as an assay substrate by incubating this DNA (20 pmol) with the cell extracts (2 mg of protein) at  $30^\circ\text{C}$  for 2 h in a 1-ml reaction mixture [50 mM Tris-HCl, 50 mM KCl, and 1 mM EDTA (pH 7.5)]. The reaction was

terminated by heating at 90°C for 5 min and then centrifuged at  $12,000 \times g$  for 10 min. Supernatants were filtered through a 0.45- $\mu$ m Millipore filter, and the filtrates were applied to immunoaffinity columns. The pass-through fractions were collected and reloaded. This procedure was repeated twice. The columns were eluted with 4 ml of 100% methanol after successive washes with 1 ml of 1 M NaCl twice, 1 ml of distilled water three times, and 1 ml of 100% acetonitrile three times. The eluants were dried out with a Speed-Vac (Sarvant, Holbrook, NY) and then dissolved in 120  $\mu$ l of HPLC eluting solution (10 mM  $\text{NaH}_2\text{PO}_4$  in 8% methanol). Aliquots (50  $\mu$ l) of dissolved samples were analyzed by HPLC fitted with an electrochemical detector (ESA 5100A) connected to a Beckman Ultrasphere ODS column (5  $\mu$ m  $\times$  4.6 mm  $\times$  25 cm). The column was eluted with elution solution at a flow rate of 0.5 ml/min.

### Statistics

Data are expressed as means  $\pm$  SE. The significances of differences were evaluated using the paired Student's *t* test.

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